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Two Mutations in the Locus Control Region Hypersensitivity Site-2 (5′HS-2) of Haplotype 19 β^s Chromosomes Alter Binding of *Trans*-Acting Factors

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There are five major haplotypes associated with sickle cell anemia (SS). Individuals homozygous for haplotypes 3 (Senegal) and 31 (Saudi Arabian) have high fetal hemoglobin (HbF) levels (15 to 30% of total hemoglobin) whereas individuals homozygous for haplotypes 17 (Cameroon), 19 (Benin), and 20 (Bantu) have low HbF levels (1 to 10%). We previously identified several point mutations in the LCR 5′HS-2 that were specific for haplotype 19 β^s chromosomes (compared to the GenBank HUMHBB reference sequence, T→G at position 8580, A→G at position 8598, and A→T at position 9114). We postulated that one or more of these mutations may alter the binding of specific *trans*-acting factors and ultimately affect the expression of HbF in these sickle cell patients. We performed gel mobility shift assays using ^{32}P-end-labeled double-stranded 19mers corresponding to each of the LCR 5′HS-2 normal (GenBank) and mutant sequences. Nuclear extracts prepared from HeLa and HEL cells were used in our experiments and neither the normal nor mutant sequence at position 8580 bound *trans*-acting factors in either nuclear extract. The 8598 mutant increased binding of Sp1; using purified protein and both nuclear extracts. HEL extracts were used to quantify the increase in Sp1 binding to the 8598 mutation and we found an increase in binding of 66 and 47%, respectively, in two shifted bands. The 9114 mutation sharply decreased binding of an unknown *trans*-acting factor by 74%. This factor was present in both HeLa and HEL nuclear extracts. © 1996 Wiley-Liss, Inc.

Key words: sickle cell anemia, locus control region, fetal hemoglobin, *trans*-acting factors, Sp1

INTRODUCTION

Five major haplotypes are associated with sickle cell anemia (SS) [1,2]. Haplotypes 3 (Senegal) and 31 (Saudi Arabian) are associated with elevated high fetal hemoglobin (HbF) levels (15 to 30% of total hemoglobin), while the other three haplotypes (19 [Benin], 20 [Bantu], and 17 [Cameroon]) are associated with lower HbF levels (1 to 10%). SS patients who have elevated HbF usually experience a less severe disease due in part to HbF interfering with the polymerization of sickle hemoglobin (HbS) [3,4]. This observation has sparked a search for the genetic and epigenetic deteriminants of differences in HbF expression among SS haplotypes.

The major regulator of β-globin gene cluster expression is the Locus Control Region (LCR) [5]. The LCR consists of at least four hypersensitive sites (5′HS-1 through 5′HS-4) far upstream of the β-globin gene cluster [6,7]. The LCR is essential for globin gene switching during development and directs copy number-dependent, position-independent expression of erythroid genes [8–10]. One hypersensitive site, the 5′HS-2, has classical enhancer activity and can confer at least 40% of the level of expression of the whole LCR [11,12]. Deletions and mutations in the LCR 5′HS-2 significantly decreased the enhancing activity of this region in both transgenic mice and cultured erythroid cells [13–17].

We and others previously identified sequence variations in the LCR 5′HS-2 specific for SS haplotype 19

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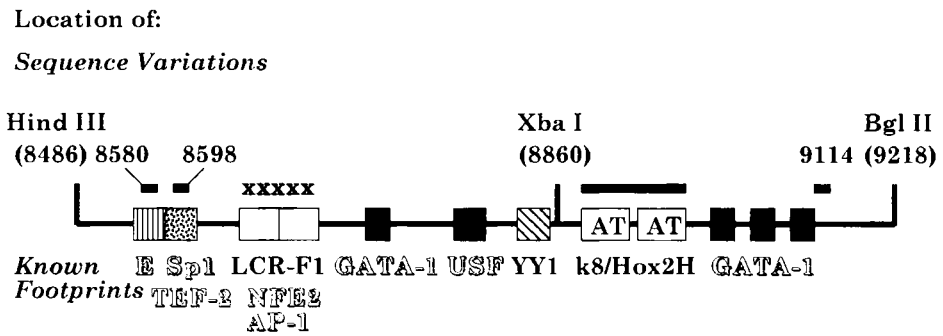


Fig. 1. The location of sickle cell haplotype 19 (Benin) sequence variations in the 5'HS-2 relative to known DNaseI footprints. The diagram, which is not to scale, is based on published data [14,16,17,22–27]. Footprints found both in vitro and in vivo are in open letters, while solid letters designate footprints found in vitro. The solid bars denote the regions of sequence variations in haplotype 19 chromosomes. The xxxxx indicates the location of maximal enhancer activity (tandem NFE-2/ AP-1 binding sites). The open uppercase "E" represents a footprint found exclusively in erythroid extracts.

(Benin) which may alter binding of *trans*-acting factors and ultimately affect HbF expression in these patients [18–20]. We previously identified the mutations relative to the ϵ-globin cap site (in parentheses), but in this work we have chosen to use the GenBank HUMHBB reference sequence to indicate the position of the mutations. Two of the three point mutations 8580 (-10924) and 8598 (-10905) are in the Hind III-Xba I core enhancer sequence of the 5'HS-2 (a region with known DNaseI footprints both in vitro and in vivo). The 8598 mutation created a consensus Sp1 binding sequence "GCCCCGCCCC" [21]. The mutation at position 9114 (-10390) is just downstream of a GATA-1 consensus sequence (see Fig. 1).

We performed gel mobility shift assays (GMSA) using ^{32}P-end-labeled double-stranded 19mers centered around the LCR 5'HS-2 normal and mutant sequences for position 8580, position 8598, and position 9114. Both erythroid (HEL) and epithelial (HeLa) nuclear extracts, as well as purified Sp1 protein were used in our experiments. Neither the normal nor mutant sequence at position 8580 bound *trans*-acting factors in either nuclear extract. The position 8598 mutation enhances binding of Sp1 and the position 9114 mutation dramatically decreases binding of a *trans*-acting factor present in both HEL and HeLa nuclear extracts.

MATERIALS AND METHODS

Cell Culture

HeLa cells (S3 strain) were grown in suspension culture in Joklik's Modified Eagle Medium supplemented with 7% calf serum, 2mM L-glutamine, 75 IU/mL of penicillin, and 75 μg/mL streptomycin. HEL cells [28] were grown attached to 150 cm^2 tissue culture flasks (Corning, Corning, NY) in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2mM L-glutamine (Mediatech), 100 IU/mL of penicillin, and 100 μg/mL streptomycin (Mediatech).

Preparation of Crude Nuclear Extracts

Crude nuclear extracts of HeLa and HEL cells were prepared essentially as described by Hennighausen and Lubon [29]. Approximately 6×10^8 HeLa and $\sim 1 \times 10^9$ HEL cells were used to prepare nuclear extracts. The protein concentration for each extract was determined using the BCA kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) (Sigma, St. Louis, MO) as a standard.

Oligonucleotide Preparation

Complementary oligonucleotides (19mers) for each normal and mutant sequence of the LCR 5'HS-2 and a 30mer with a Sp1 consensus binding site [30] were synthesized on an Applied Biosystems (Foster City, CA). 380B DNA synthesizer. A 22mer with a Sp1 consensus binding site was a gift from Promega Corporation (Madison, WI). The sense strand of each oligonucleotide used in this study is listed in Table I. Complementary oligonucleotides were annealed in TE (10mM Tris-HCl, 1mM EDTA, pH 7.5) by heating to 95°C followed by slow cooling to room temperature. After annealing, each double-stranded oligonucleotide was labeled on both 5' ends using T4 kinase (New England Biolabs, Beverly, MA), and γ-^{32}P-dATP (7,000 Ci/mmol) (ICN Biochemicals, Irvine, CA) to a specific activity of $\sim 1 \times 10^5$ cpm/ng. Labeled oligonucleotides were purified using Sephadex G-50 pre-packed columns (Pharmacia, Piscataway, NJ). The oligonucleotides were stored at −20°C until use.

Gel Mobility Shift Assays (GMSA)

GMSA were performed using crude nuclear extracts as follows: 9μg of crude nuclear extract from HeLa or

TABLE I. Sense Strand of Double-Stranded Oligonucleotides Used in Gel Mobility Shift Assays

Sequence	Position* or Reference	Designation
5' CAGGCCCC ***T*** GTCGGGGTCA 3' Normal	8572 to 8590	8580N
5' CAGGCCCC ***G*** GTCGGGGTCA 3' Mutant		8580M
5' AGTGCCCC ***A*** CCCCCGCCTT 3' Normal	8590 to 8608	8598N
5' AGTGCCCC ***G*** CCCCCGCCTT 3' Mutant		8598M
5' GGCATGAAA ***A*** CAGGAAAAG 3' Normal	9105 to 9123	9114N
5' GGCATGAAA ***T*** CAGGAAAAG 3' Mutant		9114M
5' GGCTGGCCAA ***GCCCCGCCCC*** TTTAGCCAGG 3'	Gumucio et al	Sp1-30
5' GCTC ***GCCCCGCCCC*** GATCGAAT 3'	Promega	Sp1-22

*Position of the sequence based on the GenBank HUMHBB sequence

HEL cells and 2μg of poly dI-dC (Sigma) were preincubated in 1X Gel Shift Binding Buffer (4% glycerol, 1mM $MgCl_2$, 0.5mM EDTA, 0.5mM DTT, 50mM NaCl, 10mM Tris-HCl, pH 7.5) for 15 min at room temperature before adding 20,000 cpm of labeled oligonucleotide. After addition of the labeled oligonucleotide the reaction (total volume of 10μL) was allowed to come to equilibrium for 15–20 min before adding Type III [31] gel loading buffer. The DNA-protein complexes were then separated from unbound oligonucleotide on a pre-run 4% polyacrylamide gel (30% acrylamide: 0.8% bis-acrylamide [BioRad, Hercules, CA]) in 1 × TBE using a Mini-PROTEAN II electrophoresis system (BioRad). Gels were run at 15V/cm for ~25 min. The gels were then dried and exposed to Kodak X-OMAT AR film (Georgia X-Ray, Athens, GA) in a cassette with intensifying screens at −80°C for the appropriate time.

For competition GMSA, a specified molar excess of unlabeled double-stranded oligonucleotide was added to the preincubation reaction as outlined above. All other components of the reaction were the same.

For the experiment where pure Sp1 protein (Promega) was used instead of crude nuclear extract, the reaction consisted of the following: ~50ng of Sp1 protein, 2μg of BSA, and 1μg of poly dI-dC were preincubated in 1 × Gel Shift Binding Buffer for 15 min at room temperature before addition of labeled oligonucleotide (20,000 cpm/lane). After addition of labeled probe, the reaction was allowed to continue for 15 min before addition of loading dye and electrophoresis as above.

Densitometry

For densitometric quantification of retarded DNA-protein complexes we used the IS-1000 Imaging System (Alpha Innotech Corporation, San Leandro, CA). Five identical binding reactions were set-up for each of the normal and mutant oligonucleotides used. Shifted bands were scanned for each binding reaction and quantified. Peak measurements were taken at the same coordinates for the autoradiogram when comparing each binding reaction. Densitometric values were analyzed using Sigma Plot (Jandel Scientific, Corte Madera, CA) and statistical differences were determined using Student's two-sided t-test.

RESULTS

The gel mobility shift assays for each oligonucleotide (Table I) are illustrated in Figure 2. Figure 2A illustrates gel shifts obtained using nuclear extracts from erythroid (HEL) cells, whereas Figure 2B shows the corresponding gel shifts using epithelial (HeLa) nuclear extracts. As evident in Figure 2A and B, lane 1, the Sp1-30mer bound Sp1 in both extracts strongly and produced several shifted bands similar to those previously described [30]. The Sp1-22mer (Fig. 2A,B, lane 2) also bound Sp1 although with less avidity. The 8598N and 8598M 19mers produced gel shifts similar to those for the Sp1-30mer and Sp1-22mer (Fig. 2A,B, lanes 3,4). The 8598M oligonucleotide bound more of a protein which appeared to be

Sp1 compared to the 8598N oligonucleotide. In lanes 5 and 6 of Figure 2A and B, the shifts for the 9114N and 9114M oligonucleotides are illustrated. It is evident from these gel shifts that some *trans*-acting factor present in both extracts binds the 9114N oligonucleotide with more avidity than the 9114M oligonucleotide. The association of this unknown protein with the 9114N oligonucleotide is specific; it is not competed away by unlabeled Sp1-30mer, but it is competed away by unlabeled self (data not shown). Our extracts gave the same shifted bands as a commercially available HeLa nuclear extract (Promega) for each oligonucleotide tested (data not shown). We also tested binding to each fragment in HEL and HeLa extracts using 150mM and 450mM NaCl in the gel shift binding buffer. There was no binding to either the 8580N or 8580M 19mers using HEL or HeLa extracts under any of the three salt concentrations tested (data not shown). We also varied the poly dI-dC concentration in our binding reactions (0–4μg), and poly dI-dC concentrations higher than 2μg did not alter the banding pattern, indicating that the poly dI-dC concentration was optimal to obtain specific DNA-protein binding conditions (data not shown).

The differential binding of HEL nuclear proteins to the 8598N vs. 8598M 19mers, and the 9114N vs. 9114M 19mers was quantified using laser densitometry of GMSA autoradiograms. We set up five identical GMSA for each of the four oligonucleotides. Each of the two shifted 8598M bands (* and ** in Fig. 2) have increased density relative to the 8598N sequence (8598N* and 8598N** vs. 8598M* and 8598M**). The increase in binding was 66% ($P < 0.005$) in 8598M* and 44% ($P < 0.01$) in 8598M** compared to 8598N* and 8598N**, respectively. The decrease in binding to the 9114M was 74% ($P < 0.00001$) compared to 9114N. The identity of this factor is unknown.

We used competition GMSA to determine if the increase in binding to the 8598M oligonucleotide was Sp1 related (Fig. 3). We used HEL extracts for all binding reactions. In Figure 3A and B we loaded 20,000 cpm/lane of Sp1-30mer and 0, 10×, 100×, or 1000× molar excess of unlabeled competitor as indicated. Figure 3A illustrates that the unlabeled Sp1-30mer competes well against itself, and an unrelated unlabeled 19mer, 9114N, does not compete away binding of Sp1. Figure 3B demonstrates that the cold 8598M 19mer competes for Sp1 more effectively than the 8598N unlabeled 19mer. In Figure 3C and D we loaded 20,000 cpm/lane of 8598M 19mer and 2.5×, 25×, and 250× molar excess of unlabeled competitor as indicated. These GMSA appear to demonstrate a non-specific shift for this 19mer, because it is not competed away by a 250× molar excess of unlabeled self. The cold Sp1-30mer displaced proteins binding to the labeled 8598M 19mer more effectively than the unlabeled 8598M 19mer itself (Fig. 3C). An unrelated unlabeled

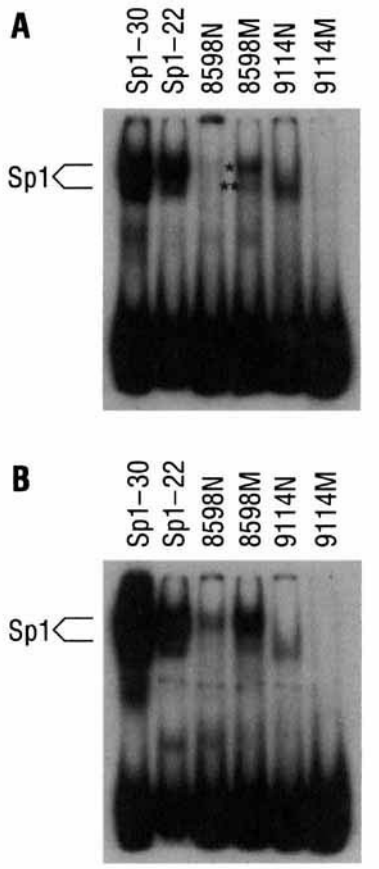
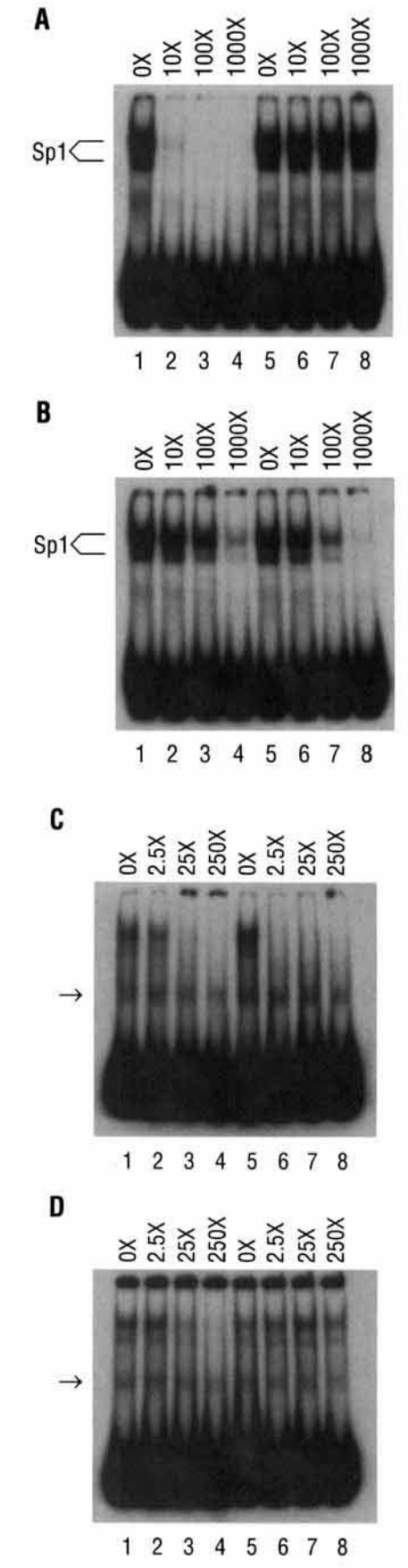


Fig. 2. Gel mobility shift assays for each oligonucleotide (Table I) using crude nuclear extracts. Each binding reaction contained 20,000 cpm of labeled oligonucleotide, 9μg of nuclear extract protein, 2μg of poly dI-dC, 10mM Tris-HCl (pH 7.5), 50mM NaCl, 1mM $MgCl_2$, 0.5mM EDTA, 0.5 mM DTT, and 4% glycerol. The oligonucleotide used in each binding reaction is indicated above each lane. A: GMSA using erythroid (HEL) nuclear extracts (* = complex 1, ** = complex 2). B: GMSA using epithelial (HeLa) nuclear extracts.

19mer, 9114N, did not displace binding at the concentrations used (Fig. 3D). The unlabeled 8598N 19mer competed away binding to the labeled 8598M 19mer less effectively than the unlabeled self. These data indicate that Sp1 is binding to the 8598M 19mer with higher avidity than the 8598N 19mer.

Finally, we used purified Sp1 in the GMSA to show that purified Sp1 would also bind the 8598M 19mer with higher avidity than the 8598N 19mer (Fig. 4). The Sp1-30mer (lane 1) and Sp1-22mer (lane 2) had a higher avidity for purified Sp1, compared to the 8598N (lane 3) and 8598M (lane 4) 19mers. These results are similar to the GMSA in Figure 2 using crude nuclear extracts. More importantly, the 8598M 19mer bound more Sp1 than did the 8598N. In addition, the 9114N 19mer which should not bind Sp1, did not (lane 5).



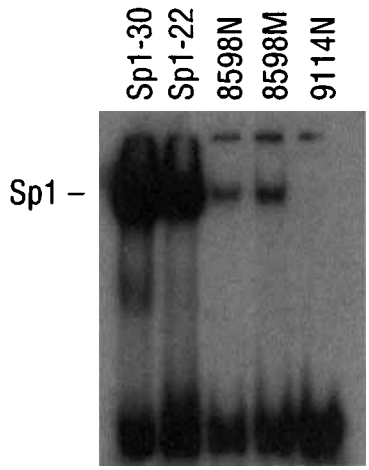


Fig. 4. Gel mobility shift assays using purified Sp1 protein. The binding reaction consisted of 20,000 cpm of labeled oligonucleotide, 50ng of purified Sp1 protein, 2μg of BSA, 1μg of poly dI-dC, 10mM Tris-HCl (pH 7.5), 50mM NaCl, 1mM $MgCl_2$, 0.5mM EDTA, 0.5mM DTT, and 4% glycerol. The oligonucleotides used in this experiment are indicated above each lane.

DISCUSSION

Many studies have documented the importance of the various LCR hypersensitive sites in the expression of the genes of the β-globin gene cluster. The LCR 5′HS-2 and -3 appear to be the most important regions of the LCR for the developmental regulation and expression of the γ-globin genes [32]. This suggests that the mutations in the 5′HS-2 of haplotype 19 SS patients could be important in the regulation of their γ-globin genes. Another piece of evidence confirming this hypothesis is that a recent sequence analysis of the LCR 5′HS-3, and -4 demonstrated that there are no haplotype-specific sequence variations which might affect binding of *trans*-acting factors [33].

In this report, the lack of binding in the GMSA for the 8580N and M (Table I) oligonucleotides is interesting

Fig. 3. Competition gel mobility shift assays using HEL extracts. GMSA were performed as in Figure 3, except a specified molar excess (indicated above each lane) of an unlabeled competitor was added to the binding reaction. A: Labeled Sp1-30 was incubated with unlabeled self (lanes 1–4) or unlabeled 9114N (lanes 5–8). B: Labeled Sp1-30 was incubated with unlabeled 8598N (lanes 1–4) or unlabeled 8598M (lanes 5–8). C: Labeled 8598M was incubated with unlabeled self (lanes 1–4) or unlabeled Sp1-30 (lanes 5–8). D: Labeled 8598M was incubated with unlabeled 8598N (lanes 1–4) or unlabeled 9114N (lanes 5–8). The arrows in (C) and (D) indicate a possible non-specific shift because it was not competed away by the unlabeled molar excess of 8598M itself at any concentration used.

because the region has a DNaseI footprint in vitro in both erythroid (K562) and epithelial (HeLa) extracts [22] and in vivo [23]. Perhaps the size of the oligonucleotide, the temperature, the pH of the binding reaction, or competition between binding sites on our 19mers affected binding.

The next mutation we studied was at position 8598 (A→G). This mutation created a perfect Sp1 consensus sequence. We have shown that the mutation enhances binding of Sp1 in crude nuclear extracts, using competition GMSA with a known high affinity Sp1 binding site, and using purified Sp1 protein. The binding of Sp1 to normal or mutant sequences in many gene promoters was previously shown to positively or negatively regulate expression of a gene. The -198 (T→C) mutation in the $^{A}\gamma$-globin gene promoter is associated with the British form of HPFH. This mutation enhances binding of Sp1 and increases expression of a linked CAT reporter gene [30,34]. In another system, overexpression of Sp1 in NIH 3T3 cells was shown to down-regulate transcription from the murine collagen α1(I) promoter which contained overlapping binding sites for Sp1 and NF-I [35]. Of more relevance to our findings is the recent work of Caterina and coworkers [17]. This work demonstrates that deletion of the Sp1 binding site (which is in our 8598N and M 19mers) from the LCR 5'HS-2 reduced expression of a linked human β-globin gene in transgenic mice from 41.5% per gene copy to approximately 10% per gene copy. This finding illustrates the importance of this sequence in the regulation of the β-globin gene, but perhaps any mutation around the Sp1 binding site (such as the 8598 [A→G]) could affect expression of the γ-globin genes in haplotype 19 SS patients.

It is interesting to note that three different oligonucleotides (Sp1-30, Sp1-22, and 8598M), each with consensus Sp1 binding sites (GCCCCGCCCC), have different binding affinities for Sp1. Perhaps this is because of the small size of the our oligonucleotide (19mer), or a result of the different sequences flanking the consensus site. These differences in binding were previously observed in another study [36]. Binding of Sp1 to short oligonucleotides was found to be at least 10-fold less than binding to a 155bp restriction fragment with the same GC box sequence. These authors also demonstrated that a restriction fragment that had the sequence "GCCCCACCCC" bound Sp1 six-fold more weakly than a perfect Sp1 consensus sequence. These data parallel the increase in binding that we saw in this experiment between the 8598N and M oligonucleotides, since the 8598N fragment has the "GCCCCACCCC" sequence and the 8598M has the perfect GC box sequence.

The other mutation we have studied at 9114 is just downstream from a GATA-1 site and markedly decreases binding of an unidentified protein found in both erythroid and epithelial extracts. This protein is not Sp1, and does not appear to be H4TF-1 [37]. We previously postulated that the mutant sequence would create a perfect H4TF-1 site (GATTTC) compared to the normal sequence at 9114. Based upon this observation we would expect increased binding to the mutant sequence compared to the normal, but instead we observed strong binding to the normal sequence and extremely weak binding to the mutant sequence and only one shifted band.

These studies implicate the LCR 5'HS-2 in regulating γ-chain expression on different genetic backgrounds, during hematopoietic stress.

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